CHARACTERIZATION OF VOLATILE ORGANIC COMPOUNDS RELEASED BY STORED GRAIN INSECTS

BY

SENTHILKUMAR THIRUPPATHI

A Thesis

Submitted to the Faculty of Graduate Studies in

Partial Fulfillment of the Requirements for the Degree of

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Department of Biosystems Engineering

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Detecting the presence of insects at low densities can avoid total deterioration of stored grains because corrective actions can be implemented early. Red flour beetle, *Tribolium castaneum* (Herbst) and Rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) are the major insect pests of the Canadian grain handling industry. Identification of the volatile organic compounds released by insects can be used to detect insects in stored grains. An attempt was made to identify the volatile organic compounds released by *T. castaneum and C. ferrugineus* by headspace analysis. The volatiles in the head space of vials with insects, insects and wheat flour, and insects and wheat, were analyzed using a GC-MS coupled with an automatic headspace sampler. Wheat with fifteen percent moisture content was used in this study along with two different insect densities. Feasibility of the automatic headspace sampler in headspace analysis was found to be positive. The sampler can do sample conditioning, absorption, trap cleaning and desorption of the volatiles into the GC-MS and speed up the process. The samples extracted at 20 strokes with 1000 µL per stroke, and desorbed at 250°C gave a clear peak of compounds.

The amount of volatiles produced by *T. castaneum* adults varied based on insect densities, the concentration of Methyl-1, 4-benzoquinone; Ethyl-1, 4-benzoquinone; and 1-Tridecene released by ten adult insects were 8.5, 9.1 and 10.6 µg/100 µL compared to 7, 8 and 4.5 µg/100 µL of Methyl-1, 4-benzoquinone;

Ethyl-1, 4-benzoquinone; and 1-Tridecene produced by five adult insects. Extreme high and low temperature leading to death produced very high amounts of volatiles compared to insects kept at 35°C. The larvae of the *T. castaneum insects* did not produce any volatiles at ambient condition as well as at extreme cold and warm conditions.

The *C. ferrugineus* adults did not produce any detectable amount of volatiles even at the higher insect density after up to 3 days. The results of the combination of *T. castaneum* and *C. ferrugineus* insects gave the same volatile organic compounds as produced by *T. castaneum* insects alone. The 1-Tridecene produced by *T. castaneum* was not reported previously in other studies.

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Dedicated to My Teachers

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Chapter 1

1. INTRODUCTION

The world population gets most of their daily energy needs from wheat and rice. The total grains produced (wheat and rice) in 2007 were 1.3 billion tonnes (Gt) (FAOSTAT, 2007), and it is very important to store these grains without any losses to feed the ever growing global population. Canada exported 32.7 Mt of food grains and oilseeds to the world market in 2008-09 crop year with wheat export alone of 17.7 Mt (CGCSTAT, 2009). Western Canada produced 24.1 Mt of wheat in 2008-2009 and Canada Western Red Spring (CWRS) wheat varieties dominated the total wheat production by occupying 70% or 17.1 Mt of the total wheat produced. Only 2.1 Mt of CWRS wheat is milled in Canada for domestic use (CWB, 2009). It is of paramount importance to protect the stored grain until it reaches the final destination to provide safe food to consumers and to get a good price for the grains produced by Canadian farmers, because the economic value of grains depends largely on the quality. Canada has a zero tolerance for insects in the food grains for human consumption. The presence of insects will cause both qualitative and quantitative losses of grains. Red flour beetle, *Tribolium castaneum* (Herbst) and Rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) are the most common insects affecting stored grain in

Canada; the presence of these insects can affect the baking quality and nutritional standards of flour, germination capacity and can produce allergens to the consumers.

Grain storage losses account for 10 percent in North America and 30 percent in Africa and Asia (Hill, 1990). Grains dried to safe moisture levels and maintained at cool temperatures reduce storage losses. Some localized areas would occur even though the average moisture content and temperature are within the safe levels. These localized areas are vulnerable to pest infestation, resulting in storage losses. The grain probe is conventionally used to detect the presence of insects inside grain bins (Campbell et al., 2002) but it is less accurate and cannot show the correct insect density (White and Loschiavo, 1986). Proper management of stored grains requires detection instruments which can accurately quantify insect density to take corrective actions to prevent further spoilage. Analyzing the amount of carbon dioxide present inside the grain bin can tell us the condition of the grain. Increase in carbon dioxide levels inside the grain bins indicates the presence of insects, mites or fungi but it cannot tell exactly the causes of spoilage (Jayas, 1995; Neethirajan et al., 2010).

The headspace analysis of stored grain to characterize the volatiles specific to different insects, mites and fungal species can be an objective method of determining the ongoing spoilage and the causes of spoilage, because each species produce unique volatile organic compounds to communicate with their own species or as metabolites. Identification of the causes of spoilage can be useful in taking proper corrective measures, because each species needs

different treatment for elimination. Identification and quantification of compounds specific to different species will be helpful in determining the insect, mites and fungi species and their densities inside a grain bin.

Headspace analysis technique involves the extraction and identification of volatile compounds present in the gas phase above the sample. Headspace analysis has been used extensively in environmental fields and also has a major presence in food and flavor industries for analyzing aroma of orange juices, fresh tomato juices, fermented dairy products, identifying lipid oxidation in foods and cooked beef to assess the quality (Rouseff and Cadwallader, 2001). Development of electronic noses and sensors need the identified volatile organic compounds released by food matrices. The volatile extraction involves use of porous polymers like chromosorb, Tenax or activated charcoal for absorbing the volatiles and the absorbed volatiles are desorbed by means of solvent or thermal desorption. The former involves washing the absorbents with organic solvents like methyl chloride, and the latter involves heating the absorbents to high temperatures (>275ºC). The solvent desorption of compounds has the possibility of causing contamination because of presence of impurities in the organic solvents and involves human error. The thermal desorption of volatile compounds are automated by auto sampler and does not involve potential of human error and less possibility for outside contamination, so thermal desorption is the most favorable method. The volatile extraction process starting from sample conditioning, volatile absorption, volatile enrichment and desorption was done separately before the arrival of automatic headspace samplers consuming

time. Today the automatic headspace sampler will do all the extraction process and can desorb the volatiles inside the GC-MS.

The desorbed volatile compounds are injected into a gas chromatograph or gas chromatograph-mass Spectrometer (GC-MS) for identification of volatile compounds in the headspace. The GC-MS is the preferred instrument for identification of volatile compounds because the mass spectrometer coupled to the gas chromatograph will produce a mass spectrum of a particular compound and we can have a more accurate result. The gas chromatograph separates the mixture of compounds present in the absorbed headspace volatiles before entering into the mass spectrometer for identification of compounds.

With the above background, the objectives of this study were:

1. to determine the feasibility of an automatic headspace sampler coupled to a gas chromatograph–mass spectrometer (GC-MS) for identification of volatile organic compounds released by stored grain insects;

2. to identify the volatile organic compounds released by stored grain insects: *T. castaneum and C. ferrugineus* when they are present inside Canadian Western Red Spring wheat and wheat flour; and

3. to quantify the amount of volatile organic compounds released by stored grain insects.

Chapter 2

2. REVIEW OF LITERATURE

2.1 Damage Caused by Stored Grain Insects

The insects, fungi, mites, rodents and birds compete with human beings for the most stable food, cereals. Losses are estimated to be approximately 15% worldwide (Reichmuth et al., 2007) (10% in North America and 30% in Asia and Africa) (Hill, 1990). About 80% of these losses are caused by insects, 10% by moulds and the remaining 10% by rodents and birds (Reichmuth et al., 2007). The losses occurring in cereals are related to 100 species of insects and mites feeding on the grain. The stored- product pests which are able to survive and reproduce even in dried food materials can cause considerable damage to the raw and processed products throughout the food chain starting from the farmer to the consumer. The estimate of these economic losses is in the range of billions of dollars worldwide.

Stored cereal grains are considered to be an ideal place for the insects to live and potentially causing significant economic losses to producers. These pests are able to satisfy their water requirement by feeding on the dry products. The infested cereals become unmarketable and often cannot be used even as

feed. The growing consumer demand for safe food forces the producer to develop proper storage techniques. The initial cost for building new storage structures and other accessories is enormous in much of the world, but when compared to the losses caused by the pests, it is small in value in a long term strategy.

2.2 Pest Management and Control

The proliferation of insects inside grain bins or other storage structures can be controlled by reducing the temperature and relative humidity. In most grain storage facilities, Integrated Pest Management (IPM) is put into practice. IPM combines measures of structural design, and thorough sanitation and cleaning with application of biological, physical and chemical methods to prevent infestation, if possible, and control the pest if it occurred despite the precautionary measures (Reichmuth et al., 2007).

Throughout the world, the stored grains are disinfested using phosphine fumigation. The phosphine is very effective and leaves no residues after use. Improved sealing techniques and stiff rules for licensed fumigators help to ensure safe use at fairly low dose. Heating the stored grains for disinfestations is a spreading technique, but the feasibility is limited for buildings of very large volume of more than about 40,000 m^3. Heating to 55°C for 24 h may be sufficient to control all insects. Also temperature below 0°C leads to complete control when applied over sufficient times of exposure (Reichmuth et al., 2007).

2.3 Need for Grain Storage System

The stored grains are still vulnerable even after maintaining them at proper temperature, moisture content and relative humidity. There are some localized areas that occur inside the grain bins even after the average moisture content, temperature and relative humidity are at safe storage levels. The spoilage of stored grains starts at a localized high temperature or high moisture content zone potentially resulting in the development of a hot spot. There are two types of hot spots, one is a fungal induced hot spot and the second is an insect induced hot spot (Sinha, 1967). These hot spots are favorable places for the insects and fungi to survive and reproduce, and the hot spot can develop at any place inside the bin. Moisture pockets will develop inside the grain bins due to moisture migration even after the average moisture content is at safe storage levels (Jayas, 1995). These moisture pockets will allow the fungi to grow and produce hot spots; these hot spots will be a favorable place for the insects to grow. High insect density of all life stages of many species were found in the hot spots of farm granaries of Manitoba and Saskatchewan (Sinha, 1961).

The identification of insect densities inside the grain bins is considered to be a mammoth task for grain storage managers. The insects are detected conventionally by placing a grain probe or trier inside the grain bins (Campbell et al., 2002). But the grain probe or trier detection is not detecting accurate insect densities and is providing improper insect numbers compared to the actual number of insects present inside grain bins (White and Loschiavo, 1986). So the current need of the grain storage facilities is a proper technique to identify the

insect densities inside the grain bin. The amount of carbon dioxide present inside the grain bin can be an indicator of the presence of pests like insects, mites or fungi (Jayas, 1995; Neethirajan et al., 2010). But with this method we cannot say that insects are present inside the bin, because it may be fungi, or mites.

The current need of storage facilities is to have a new method which can tell the insect densities and insect species. The need for knowing the insect species will be helpful in eliminating the insect species by using the proper method suitable for that insect. Monitoring the volatile compounds released by grains by headspace analysis can be a good method for identification of insect species, and if quantified, the amount of volatiles produced by insects can provide the actual insect density present inside the grain bin.

2.4 Canadian Grain Storage System

T. castaneum and *C. ferrugineus* are the most common insects occurring in stored grains in western Canada. *Sitophilus oryzae* (L.), *Sitophilus granaries*(L.), *Plodia interpunctella* (Hubner), *Oryzaephilus surinamensis* (L.), and *Rhyzopertha dominica* (F.) are other insects occurring in stored grains in Western Canada (Sinha and Watters, 1985). Canada produced 24.1 Mt of wheat during the 2008-2009 crop year, this huge amount of wheat has to be stored properly until it reaches the consumer. The moisture at which wheat is harvested is 20-35% at temperatures around 10-35°C. This moisture content level and temperature allow for insects and mold damage. To keep the wheat without

damage the initial moisture content should be reduced to safe storage levels of 13 to 14% moisture content by air drying and cooled to low temperatures using aeration in western Canada.

Canada is the second largest exporter of wheat to the world market. Canada practices zero tolerance for insect pests in grains for human consumption, and has an excellent reputation worldwide for grain quality. The economic value of the grains is determined by the quality of the grain. The grains damaged by insects have lesser value and sometimes grain may not be fit for human consumption due to higher infestation. So it is important to preserve the stored grains from pests to provide safe food for the consumers and get high economic returns for the Canadian grain producers.

2.4.1 *Tribolium castaneum*

T. castaneum is red-brown in color (Figure 2.1). The insect is 3 to 4 mm long. The female lays 2 to 18 eggs daily loosely into the feeding substrate, altogether 1000 eggs within 400 days. The larva hatch after 3 to 14 days. The larva pupates freely after 4 moults in the substrate at a protected location. The development from egg to adult lasts about 93 days at 22°C. The beetles do not fly at temperatures below 25°C even though their wings are fully developed. They do not survive at low temperatures. At 7°C they survive for about 25 days and at -6°C less than one day. Mostly they are protected against quick cooling or heating by the insulation of their feeding substrate. The temperature range for development is 22-40°C with an optimum at 32-37°C (Reichmuth et al., 2007).

Figure 2.1 *T. castaneum* (Herbst), the red flour beetle

This beetle is also carnivorous and attacks other stored-product insects. In competition with *Tribolium confusum*, this species is slightly weaker. These insects can live in the relative humidity range of 10% to 95%. The optimum relative humidity is between 70 and 90% (White et al., 1995).

This insect typically feeds on seed germ and then endosperm. Due to its high fecundity and relatively short development time, the red flour beetle can cause losses of large masses after heavy infestation. Therefore, it is a severe pest in the food processing industry. Infested flour turns slightly rose-colored and picks up a specific smell and bitter taste. Smell and taste of infested bakery products or bakery products produced by infested grains are negatively influenced. These changes are caused by quinones excreted by the beetles that have communication properties and may also be carcinogenic according to recent investigations. In comparison, these changes in quality are even more extensive than the direct quantity losses. Heavy infestations in stored grain will occur only when the grain is warm. Most frequently this species appears together with other pest insects. It usually infests the damaged grain kernels, grain products and grain flour (Lhaloui et al., 1988). It has a tendency to attack damaged grain, but it can eat whole wheat, consuming the germ first and moving towards the endosperm (Agriculture Canada, 1981).

Control of this very frequent pest insect in the milling industry is not easy. The beetles may live hidden in or underneath milling machinery, sieving machines or in flour which has not run out of the silos and sticks somewhere to the walls. From these places they may infest the freshly produced flour. At

present fumigation with sulfuryl floride in emptied factories and mills as well as application of heat of about 55°C for two to three days seem to be the most appropriate control measures, accompanied with very thorough cleaning to avoid quick reinfestation. The phosphine fumigation is another effective method of disinfestations of the *Tribolium*-infested grain bins.

2.4.2 *Cryptolestes ferrugineus*

C. ferrugineus adults are about 2 mm long (Figure 2.2). They are red-brown in color with 1 mm long antennae. The insects usually lay eggs (0.6 mm in size) in between the kernels of the infested substrate. Then the egg develops into a larva of 4 mm length and it is yellow-whitish with two hooks at the rear end. The larva enters the germ of the seed and develops into the pupal stage after four larval molts and is webbed close to the germ of the kernel (Reichmuth et al., 2007). These insects occur frequently in stored grains. They usually damage the germ, and eat the remaining part. The insect needs five weeks to develop from egg to adult insects at summer temperature and need up to 12 weeks development at low temperatures. The temperature of the centre of infestation will rise when there is heavy infestation by rusty grain beetle and the complete development will require only three weeks instead of five weeks. In North America, the rusty grain beetle is more predominant in grains bins and is more problematic to the storage managers. If control measures are not carried out carefully after finding ongoing infestation, grain may become lumped together deep in the bulk and become unsuitable for transport by conveyor belt or auger because of associated caking by molds.

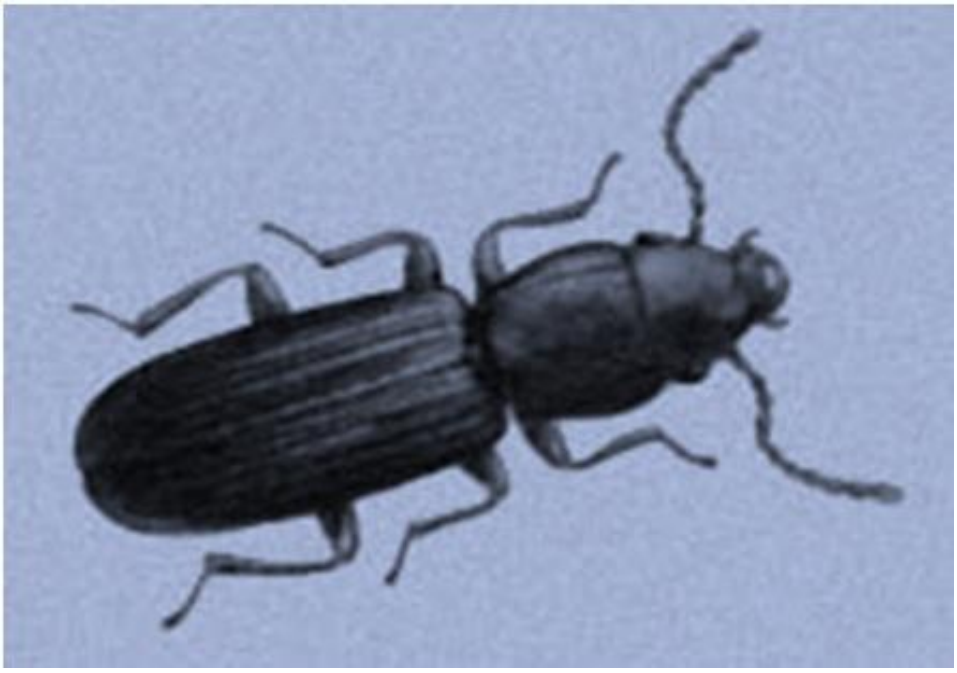


Figure 2.2 *C. ferrugineus* (Stephens), the rusty grain beetle

This situation is considered as a disaster in grain silos, because these hot spots cannot be moved through the cone and opening of the bin. The primary method of preventing any problem with this beetle during storage of grain and other products is thorough sanitation particularly of cracks and crevices where residual insects may hide prior to new loading of grain. Lowering the temperature of grain to 15°C avoids development of insects.

The storage managers have to be more careful during warm periods because there will be rapid development of insects. Immediate cooling of the infested area may interrupt the dynamics of the population development. Cooling can be used for disinfestations if aeration is done in winter. At present only phosphine is commonly used to kill all developmental stages within one week during much of the year (Reichmuth et al., 2007). There are other methods available to eliminate the insects.

2.5 Headspace Analysis

Headspace analysis involves the extraction of analytes above a liquid or solid and in between a non-volatile liquid or solid phase and vapor phase and this extraction method is generally known as vapor-phase extraction. This method is simple, sensitive, and without non volatile residues. The traditional methods involving extraction, absorption, precipitation, distillation carry more non-volatile residues, and impair the performance of volatile identification instruments like gas chromatographs and high performance liquid chromatographs. There are

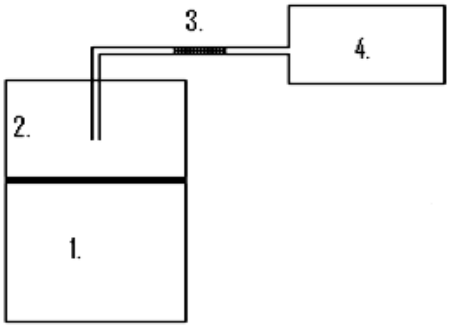
two forms of headspace analysis: static and dynamic. These are discussed in sections 2.5.1 and 2.5.2, respectively.

2.5.1 Static Headspace Analysis

Static headspace (Figure 2.3) analysis is one form of headspace analysis, which involves a single step gas extraction procedure above a solid or liquid sample. In static head space analysis, the sample to be analyzed is placed in a closed container and is placed in a heated chamber, to force the residual organic compounds to the headspace. A period of time is needed to reach a equilibrium of the solid or liquid and vapor phase. This equilibrium occurs when the rate of volatiles moving out of the samples equals to that of the volatiles moving back into the samples. After it comes into equilibrium with its vapors at a predetermined temperature, the volatiles present in the headspace were collected in the past using a syringe, and then using porous polymers. Now people have started using SPME fibers to collect the volatile compounds. These absorbed volatiles are then introduced to GC/GC-MS for identification and quantification. Static headspace analysis is used to determine partition coefficients or the partitioning of selected known compounds, rather than for the analysis of all the volatiles in a sample of interest.

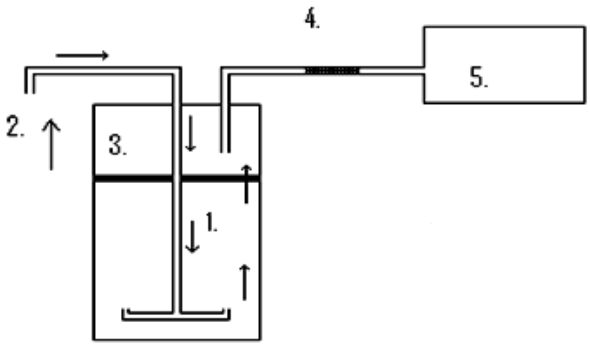
2.5.2 Dynamic Headspace Analysis

We can classify the dynamic headspace (Figure 2.4) as a purge and trap method. This method is devised in such a way to overcome the limitations in the static head space analysis. Here the sample to be tested is kept in an air tight jar fitted with two ports, one for the purge gas and another one to trap the volatiles.



1. Liquid or solid sample, 2.headspace, 3.absorbent and 4.GC-MS

Figure 2.3 Static headspace analysis



1. Liquid or solid sample, 2.Purge gas, 3.headspace, 4.absorbent, 5. GC-MS

Figure 2.4 Dynamic headspace analysis

The purge gas usually used is 99.9 % pure nitrogen; it is used because it is an inert gas. The purge gas removes the volatiles present in the intraganular spaces of the samples resulting in more concentrated volatiles, the trapping is done in the same way as used for static head space; syringe, absorbents, or by SPME fibers. Dynamic sampling provides more volatiles at trace levels.

2.6 Absorbents

The headspace analysis started by using a syringe to collect the volatiles in the headspace of the samples without enrichment of volatiles. The porous polymer absorbents, Solid Phase Micro Extraction (SPME) and Stir Bar Sorptive Extraction (SBSE) are frequently used for the absorption of volatiles in the headspace and these concentrate the volatile organic compounds.

2.6.1 Solid Phase Micro Extraction Fibers

SPME fiber is a new and efficient sample extraction absorbent invented by Pawliszyn in 1989. SPME has an absorptive layer which absorbs solutes above or from liquid or solid sample in both static and dynamic headspace. The desorption of the solutes can be done with both thermal and liquid desorption methods.

2.6.2 Stir Bar Absorbents

Magnetic stirring bar absorbents are sold under the name “Twister” and the technique is known as Stir Bar Sorptive Extraction (SBSE). The extraction mechanism and advantages of SBSE are similar to the SPME, but with a higher ratio of coating phase leading up to 100 times lower detection limits than SPME.

The SPME fiber was used in detecting the volatile compounds present in the headspace of wheat flour (Maeda et al., 2008).

2.6.3 Porous Polymer Absorbents

Porous polymers are used to absorb and preconcentrate the analytes present in the headspace of the sample. The commercially available porous polymers are Chromosorb 102, 103, or 105; Porapak Q; Tenax TA; Hayesep Q; and activated charcoal. The Chromosorb and Tenax absorbents are most commonly used for food and flavor analysis.

2.7 Desorption of Volatile Organic Compounds

The desorption of analytes from the porous polymers, SPME fibers, Twisters can be achieved by either elution with a solvent (solvent desorption) or rapid heat treatment (thermal desorption).

2.7.1 Solvent Desorption

The compounds absorbed by the porous polymers are dissolved by organic solvents like, hexane, pentane, diethyl ether, and dichloromethane.

2.7.2 Thermal Desorption

Compounds absorbed by SPME fibers, porous polymers are usually desorbed by thermal desorption methods. The absorbents are heated to a temperature of up to 350°C so that the volatiles absorbed from the headspace tend to move inside the gas chromatograph for identification of compounds. The

recommended desorption temperature is 250°C. Some modern GC-MS have the built-in provision to desorb the volatiles.

2.8 Instrumentation for Identification of Volatile Organic Compounds

2.8.1 Gas Chromatography

Gas chromatography is an analytical method for separating chemical compounds in a liquid or gaseous mixture. The samples injected into the injector are pushed inside the column by a carrier gas, nitrogen or helium. The carrier inert gas is called the mobile phase and the column is called the stationary phase. The compounds present inside the sample travel inside the column at different speeds based on their physical and chemical attributes and reach the detector. The flow chart of various parts of a GC is shown in the Figure 2.5. Gas chromatograph is used to separate the different compounds present in the sample, and there are different kinds of detectors used to detect the compound coming out of the gas chromatograph. The most common detectors are Flame Ionization Detector (FID) and Thermal Conductivity Detector (TCD).

2.8.2 Mass Spectrometry

Mass spectrometry (MS) is a method which can give qualitative and quantitative information about the elemental composition of inorganic and organic compounds. The mass spectrometer uses an ion source to charge a molecule and produces a parent ion and ion fragments of the molecule and sorts these ions and fragments based on their mass to charge ratio to form a mass

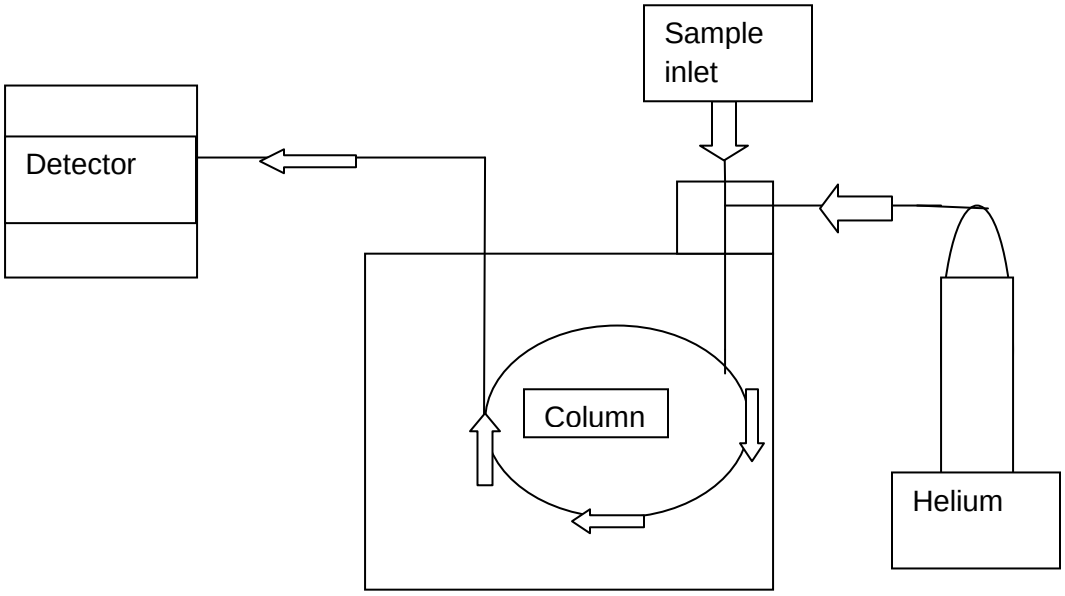


Figure 2.5 Gas chromatography

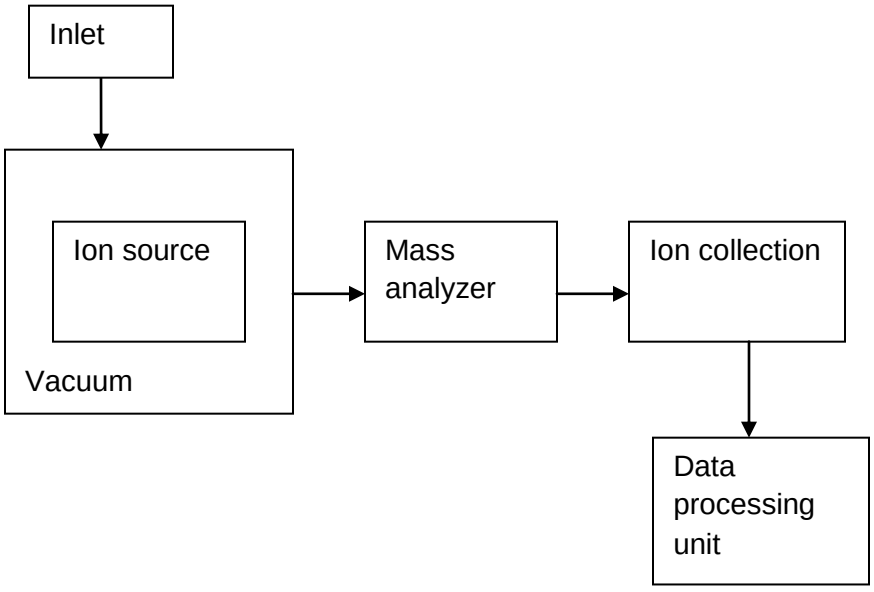


Figure 2.6 Mass spectrometry

Spectrum (Figure 2.6). The mass spectrum can characterize a particular compound based on different kinds of ions. The main advantage of using mass spectrometry is its increased sensitivity in identifying unknown compounds compared to other analytical techniques.

2.8.3 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is the analytical technique, which can be considered as a GC with MS as the detector or as a mass spectrometer with a GC as a molecule separator in a mixture before ionization of separate molecules. The GC-MS results can be very accurate when compared to GC or MS machines, because any compounds that have similar physical and chemical properties can come out of the GC at same time, and we recognize both compounds as a similar compound, but by the presence of the MS this problem can be eliminated by the production of a mass spectrum.

2.9 ITEX Method coupled to GC-MS

In-Tube Extraction (ITEX) is a new method which combines both the advantages of static and dynamic headspace analysis. The ITEX uses a syringe with absorbents placed between syringe and its needle to collect the volatiles in the headspace. The collected volatiles are thermally desorbed into the GC or GC-MS for identification of the volatiles. The syringe-only concept provides transparent sample handling and there is no sample loop, transfer lines, or switching valves. It does not need any GC injector modifications, and there is no

need for cryofocussing. The sample extraction technique starting from sample conditioning, absorption, and desorption are fully automated which saves time for the analysis of samples.

2.10 Applications of Static Headspace Analysis

In the food industry, static headspace analysis has been widely used in assessing flavors in foods. Marsili (2001) used SPME –static headspace technique to identify the off-flavors produced during oxidation of foods. The static headspace analysis coupled to the flame photometric technique was used to determine the 13 sulphur compounds present in wine, the results obtained showed that the static headspace analysis was the best way for analyzing both white and red wine for their sulphur compounds (Mestres et al., 1997).

The aroma of heated and non heated orange juice was analyzed by the static headspace –SPME method (Rouseff and Cadwallader, 2001). Fereidoon (2001) identified the lipid oxidation of foods using the static headspace method. The aldehydes are secondary breakdown products of unsaturated lipids. Polyunsaturated lipid fatty acids of the omega-3 and omega-6 families are highly susceptible to oxidation, so they monitored the oxidation process by using static headspace analysis. The most important process in the pharmaceuticals industry is the identification of toxic residual solvents present during the preparation of drugs. The static headspace analysis has been applied successfully for prompt identification of the residual solvents present during the preparation of drugs (Camarasu, 2000). There is a large development for static

headspace applications in forensic, biological and clinical analysis. Static headspace analysis is used for the analysis of alcohol content in blood and low molecular-weight materials in the blood, as well as in arson and fire investigations by collecting the fire debris which is critical in forensic analysis (Ren and Bertsch, 1999). The excellent sensitivity, reproducibility and stability make static headspace sampling a method of choice in forensic analysis. The liquid accelerants used for arson can be identified by analyzing the fire debris by the static headspace method. The volatile compounds produced by all the accelerants were found and these identified compounds can be helpful in detecting the accelerants used for the arson. The method was considered to be a more rapid and effective way of identifying the cause of the fire (Stephen and Pawliszyn, 1996).

2.11 Applications of Dynamic Headspace Analysis

The dynamic headspace analysis was applied (Kim and Morr, 1996) to identify the volatile compounds responsible for light-activated flavor in milk, and it has been proven that dynamic headspace analysis can detect the volatiles produced in 12 h whereas a sensory panel cannot detect the flavor change after 12 h. The amount of furaneol given out by the tomato can be an indicator of tomato ripened by ethylene or ripened on the plant itself. Dynamic headspace sampling was used to find the amount of furaneol produced by the tomato and the results showed that the amount of furaneol was less in ethylene-

ripened tomatoes compared to plant ripened tomatoes (Buttery et al., 2001). Johns et al. (2005) used dynamic headspace analysis to identify 1,1,1-trichloroethane, trichloroethanol, and trichloroacetic acid in urine samples. The studies using dynamic headspace analysis gave higher sensitivity compared to static headspace analysis. Volatile compounds analysis of extra virgin olive showed that static SPME is simple and rapid, but dynamic headspace analysis is efficient in collecting higher number of compounds (Kanavouras and Hemandez, 2006).

2.12 Headspace Analysis in the Grain-Handling Industry

The headspace analysis in the grain industry started by using syringes for extracting volatiles, and then evolved to porous polymers. Today the automatic headspace samplers are widely used to collect the headspace volatile organic compounds. The headspace technique was used to identify the different varieties of maize, wheat, rye, and triticale. The results showed that the compounds were similar qualitatively but except for two wheat varieties, quantitatively different (Hougen et al., 1971). Volatile fungal metabolites were collected and identified for early detection of spoilage using purge and trap headspace volatile extraction and compounds were analyzed using GC-MS. The fungi *Aspergillus amstelodami,* (Thom and Church) *Aspergillus flavus* (Link), *Penicillium cyclopium* (Westling), *and Fusarium culmorum* (Sacc.) were grow on wheat medium. The total sampling time was 90 min to collect the volatiles. The

fungi produced 2-methylfuran, 2-methyl-1-proponal, and 3-methyl-1-butanol during early stages of growth (Borjesson et al., 1989).

Three volatile organic compounds, 3-methyl-1-butanol, 1-octen-3-ol and 3-octanone were produced by wheat at 15.6% and 18.2% moisture content in ventilated and non ventilated bins as identified by headspace analysis. These volatiles can be an indicator of incipient spoilage (Sinha et al., 1988). The time required to collect the volatiles was 17 h. The same methodology was used to collect the volatiles produced by fungal infected damp wheat at 20 and 25% moisture content, the results shows the same three volatile compounds identified by Sinha (Tuma et al., 1989). The studies done on fungal volatiles shows that the volatiles produced are the same, but their concentration increased by increase in moisture content of wheat. Volatile defensive secretions of *Tribolium castaneum* were identified using SPME fiber extraction and GC-MS, the insects placed in the empty vial without agitation produced trace levels of volatiles, whereas insects agitated for 30 s produced large amounts of Methyl-1, 4-benzoquinone; Ethyl-1,4-benzoquinone; and 1-Pentadecene and it was also reported that the amount of volatiles produced at increased temperature of 90°C were equal to the agitated insects placed in the empty vials (Villaverde et al., 2007).

Male tenebrionid beetles produce sex pheromones which were identified by the SPME headspace-GC-MS method by stimulating the insects by spraying water on them. The only volatiles found using this method was 1-Tridecene. The results were compared with the extraction of the sperm from the aedeagal

gland secretion and pygidial gland secretion obtained from dissected male insects. The results obtained from the glands were similar to the headspace analysis using the SPME fiber (Geiselhardt et al., 2008).

2.13 Summary

Headspace analysis using an automatic headspace sampler coupled to GC-MS can be an objective method for analyzing the volatile organic compounds released by stored-grain insects. The identified compounds can be helpful in development of sensors for monitoring stored grains. There are various studies in the literature to identify the volatiles released by insects alone, and in some studies wheat was used as a substrate, but the volatiles were collected and concentrated over a long time. This study is framed to identify the volatiles released by insects immediately after they enter stored grains. This information will be helpful in determining the incipient spoilage occurring in stored grains.

Chapter 3

3. MATERIALS AND METHODS

3.1 Insects Culture Preparation

Commercial wheat flour of 1 kg thoroughly mixed with 10 g of Brewer's yeast was used as a medium for rearing *T. castaneum*. *Cryptolestes ferrugineus* were reared on 2 kg semi-ground CWRS wheat (*Triticum aestivum L.*). Both insects reared on the media were obtained from individuals received from a farm in Landmark, Manitoba in 1998 and the insects used in this study were mixed sex and maintained at 30°C and 70% relative humidity.

3.2 Grain Sample preparation

The CWRS wheat is the largest produced wheat class in Canada (CWB, 2009), so we used CWRS for this study. The wheat was conditioned to a moisture level of 15% (wet basis) by adding a calculated amount of distilled water to wheat of initial moisture content of 12.5%. Moisture content was determined by oven drying 10 g of wheat in triplicates at 130±2°C for 19 h (ASABE, 2008).

3.3 Volatile Collection

3.3.1 Automatic Headspace Sampler

Volatile organic compounds present in the headspace of the vials were collected using an auto sampler (CTC Analytics AG, CH-4222 Zwingen, Switzerland). The auto sampler was automated by using Cycle Composer PAL 1 software (CTC Analytics AG, CH-4222 Zwingen, Switzerland). The auto sampler has three main parts, the sample holder, sample conditioner and the ITEX head as shown in Figure 3.1. The sample holder can hold 32 vials at a time, and a sample conditioner which has a provision to place the vials and can heat the vials starting from 30°C to 200°C. The ITEX head when automated collects a vial from sample holder, places it in the sample conditioner for the programmed time and starts collecting volatiles in the headspace of the vial. A microtrap with Tenax absorbent was placed between the headspace syringe and syringe needle. The part of the gaseous phase of the pre-conditioned sample vial was pumped by syringe repeatedly through the microtrap. The volatiles absorbed in the microtrap were thermally desorbed into the GC injector for identification of compounds. The microtrap was rapidly flash-heated and the analytes reached the GC column as a narrow band. No cryofocussing was needed to obtain sharp peaks. To prepare for the next extraction, the hot trap was re-conditioned outside the injector with clean purge gas. This system allowed rapid, simple and efficient extraction of volatile organic compounds.

Figure 3.1 Automatic headspace sampler

1. ITEX head, 2. Sample holder, 3. Sample conditioner

3.3.2 Volatile Extraction

Samples present in the vials were kept in the sample conditioner for 5 min at 35°C to simulate the real grain bin which can be at an average temperature of 35°C at harvest. Number of extraction strokes and extraction volume required for this study was determined in a preliminary study by running samples at different extraction strokes and extraction volumes. It was found that 20 extraction strokes at the rate of 1000 µL per stroke gave a clear peak in the chromatogram, and was helpful in the identification of the volatile organic compounds. The extraction speed was kept at 100 µL per second, the factory default value. After the extraction the absorbed volatiles were desorbed into the gas chromatograph injector by thermal desorption at 275°C.

3.4 Identification of Volatile Organic Compounds

A Varian CP-3800 gas chromatograph coupled to a Varian 320-MS mass spectrometer (Palo Alto, California, USA) was used for identification and quantification of volatile organic compounds released by stored-grain insects. The gas chromatograph was fitted with a Varian factor four capillary column (VF-5ms 0.25 mm x 0.25 µm x 30 m), and the column oven was programmed to start at 40°C for 3 min, increased to 80°C at the rate of 5°C per min, then increased to 150°C at the rate of 20°C per min, and finally to a temperature of 250°C at the rate of 30°C per min and a hold of 10 min at 250°C , thus the total run time was 27.83 min. The thermally desorbed mixture of volatile compounds entered the injector and reached the GC column. Each compound eluted at different retention times based on their chemical properties. These separated

compounds entered into the mass spectrometer for ionization and compounds were identified based on their fragments using their mass to charge ratio (mass spectrum) and the mass spectrum was compared with the National Institute of Standards and Technology (NIST) library to identify the compound. The mass spectrometer acquisition range was kept at 20 to 300, and electron ionization was used at 70eV for the fragmentation of compounds.

3.5 Experimental Design

The experiment was designed to identify the compounds released by insects and to find the difference in the amount of volatiles based on the insect numbers. Three replications were done to find the average amount of volatiles released by five or ten adult *T.castaneum* insects. The insects were transferred into the 20 mL sample vial which has 2.5 g of wheat flour as substrate and was tightly sealed. The volatiles were extracted after 24, 48 and 72 h, because preliminary studies showed that there were no volatile compounds present in the headspace of the vials after 18 h of transferring the insects. The volatiles of *T. castaneum* were collected after placing the insects in vials with wheat at 15% moisture content. The same method was followed for *C. ferrugineus* with 25, 50 and 100 adults per vial. The insect combination of *C. ferrugineus* and *T. castaneum* was used at the ratio of 10:1. The larvae of *T. castaneum* were also used to determine the volatiles produced by larvae. Fifteen larvae were placed in the empty vials to collect the volatiles.

Figure 3.2 Experimental setup of the volatile extraction system gas chromatograph and mass spectrometer

1. Automatic headspace sampler, 2. Gas chromatograph 3. Mass spectrometer 4. Data processing unit 5. Helium cylinder.

3.6 Quantification of Volatile Organic Compounds

The amount of volatile compounds received from the GC-MS was represented as area under the peak. To change the results to concentration in µg/100 µL level a calibration peak was created. The calibration peak for benzoquinone was created, because the two major volatile compounds Methyl 1-4, benzoquinone; and Ethyl 1, 4-benzoquinone were not available commercially and benzoquinone was assumed to be similar to these compounds as assumed in a previous study conducted on *T. castaneum* (Villaverde et al., 2007). Benzoquinone (0.050g) was placed in the sample vial which was tightly sealed. The sample vials with benzoquinone were placed in the automatic headspace sampler and the volatiles were extracted as per the same procedure followed for insects except for the volume of volatiles extracted to create the calibration peak. The amount of volatile extraction was done with 100,150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1000 µL, because the area under the peak we got at 1000 µL was much higher than the amount of insect volatiles. The calibration peak for benzoquinone was created by leaving the first two data points (4.28 and 6.42 µg) in order to get the best fit line and to avoid negative trend line as shown in Figure 3.3. The concentration of benzoquinone for 100 µl of sample was calculated to be 4.28 µg. The calibration peak for 1-Tridecene was created by placing 1 µL of 1-Tridecene (99.9% pure). The amount of volatiles extracted was 10, 25, 100, 200, 400, 600, 800, 1000, 1500, 2000, 3000 µL. The calibration peak of 1-Tridecene was shown in Figure 3.4. The concentration of 1-Tridecene for 100 µL was 7.02 µg.

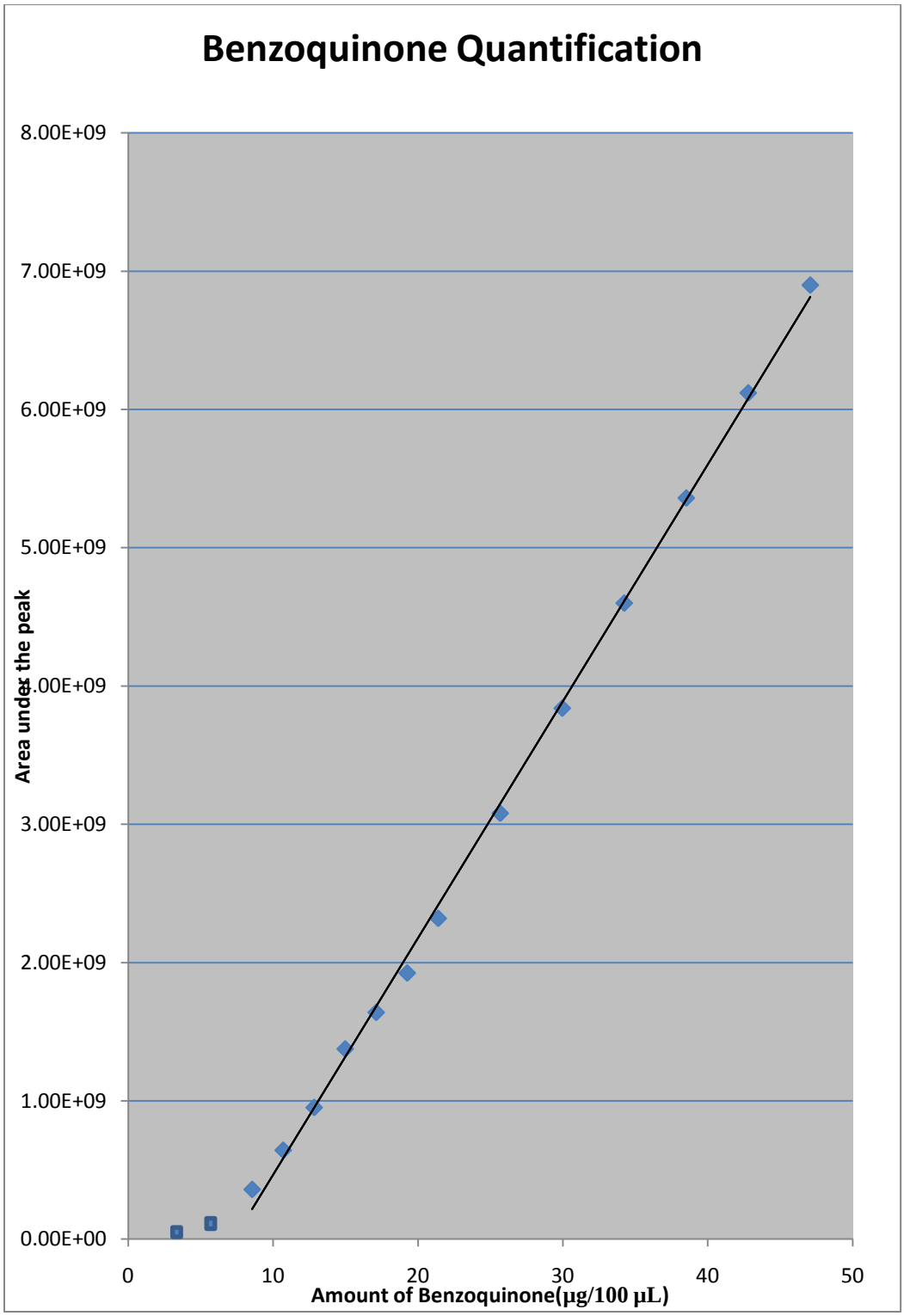


Figure 3.3 Quantification chart of benzoquinone

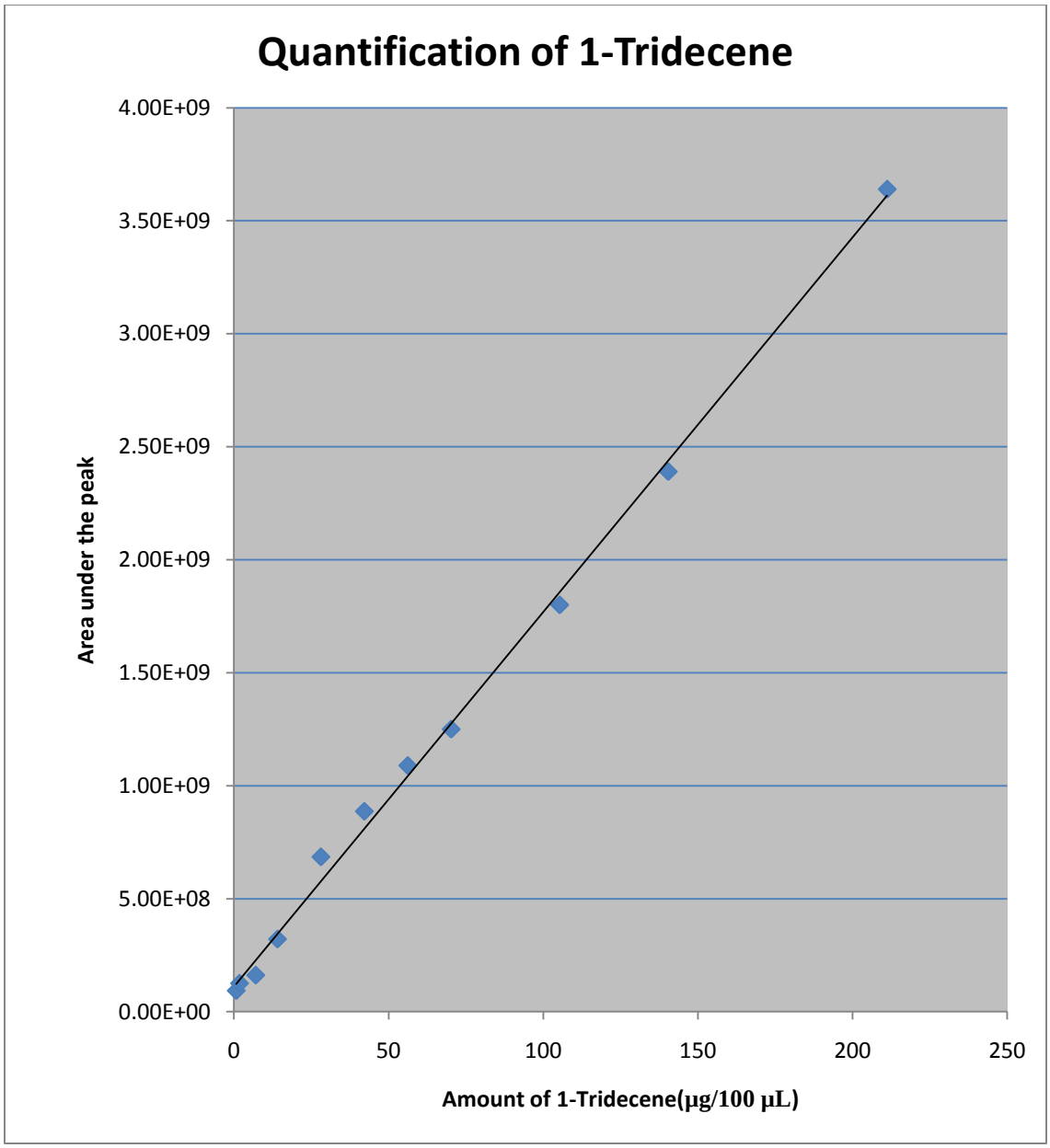


Figure 3.4 Quantification chart of 1-Tridecene

Chapter 4

4. RESULTS AND DISCUSSION

4.1 Optimization of the Automatic Headspace Sampler

The samples collected by the automatic headspace sampler with 20 strokes at a volume of 1000 µL gave a clear peak when analyzed by GC-MS. The optimum speed of absorption was set at 100 µL/s. The desorption temperature at 250°C removed all the traces from the absorbent, whereas anything below 250°C left some traces and the trace amount was identified by running a blank immediately after running the insect sample. The syringe starting temperature at 60°C gave a good degree of absorption as recommended by the ITEX product manual. The incubation time of 5 min at 35°C for some samples provided an easy way to simulate insect activity in a typical grain bin. The sample vials were closed tightly to make sure the volatiles did not escape and the vials filled half way to make sure the syringe was not hindered by the wheat or wheat flour present in the vial.

The GC-MS programming was finalized by running trials which involved different column oven temperature programs and different mass spectrum

analysis. The GC column oven temperature started at 40°C and ended at 250°C because most of the compounds had boiling points below 250°C. The mass range was set between 40 - 300 because most volatile organic compounds are within this range of molecular mass.

4.2 Wheat Flour Studies

The insects placed in the vial with 2.5 g of wheat flour did not provide any peak when the gaseous part of the headspace of vials was extracted. Then we extracted the samples headspace after 6, 12, 18, 24, and 48 h. The chromatographic results at the end of 48 h did not give any results except for one sample out of six. The sample headspace extracted after 72 h gave peaks that were not present in the control samples. The identified compounds were Methyl-1, 4-benzoquinone; Ethyl-1, 4-benzoquinone; and 1-Tridecene. The same three compounds were present in the sample which gave a peak after 48 h. The mass spectrum of the compounds identified was compared with NIST Library, and the accuracy was more than 95% for all three compounds. Among the three compounds produced, Ethyl-1, 4-benzoquinone was higher than Methyl-1, 4-benzoquinone and 1-Tridecene was at lower amount compared to the other two compounds in vials with 5 adult insects and levels were higher for 10 adult insects when compared to other two benzoquinone derivatives. The average concentration of volatiles produced by five and ten insects are given in Table 4.1 as area under the peak and quantified to µg/100 µL. The reasons for not having any volatile compounds in the headspace immediately after putting the insects in vials is because the insects were inside the wheat flour immediately after the

Table 4.1 Amount of volatiles produced by *T. castaneum* adults in units of area under the peak or concentration (µg/100 µL).

No of Insects	MBQ		EBQ		1-Tridecene	
	Area under peak	Concentration (µg/100 µL)	Area under peak	Concentration (µg/100 µL)	Area under peak	Concentration (µg/100 µL)
5	8.76E+07	7	1.19E+08	8	9.52E+07	4.2
10	2.34E+08	8.5	3.36E+08	9.1	2.37E+08	10.6

MBQ-Methyl 1-4, benzoquinone, EBQ-Ethyl 1, 4-benzoquinone

transfer of insects from culture to vials. The volatiles produced by the insects were absorbed by the wheat flour itself. This was confirmed by heating the samples to a higher temperature of 50°C after removing the insects from the vials and heating the vials with wheat flour alone. The one sample which gave out volatiles after 48 h was visually inspected and the insects were present on top of the wheat flour, whereas in the remaining 5 samples the insects were hidden inside the wheat flour. The chromatographic results for the 10 insects sample after 24, 48, and 72 h, are shown in Figure 4.1.

The wheat flour with five and ten insects turned to pale rose color because of quinine contamination, similar to reports by Hodges et al. (1996). The volatile organic compounds Methyl-1, 4-benzoquinone, and Ethyl-1, 4-benzoquinone were previously observed by Villaverde et al. (2007) using SPME fibers with insects alone. In the same study they reported one more compound Pentadecene which is reported in their study, and they termed these volatiles defensive secretions.

The 1-Tridecene we observed in our study was not previously reported in any studies related to *T. castaneum*, but the studies carried out with male *Parastizopus transgariepinus* (Geiselhardt) gave out 1-Tridecene and they labeled it as a male sex pheromone produced from sperm of this species and extracted the secretion and analyzed it using GC-MS. This confirmed the presence of 1-Tridecene (Geiselhardt et al., 2008). *Parastizopus transgariepinus* and *T. castaneum* are different species but are from the same family (Tenebrionidae). The 1-Tridecene may be considered to be a sex pheromone.

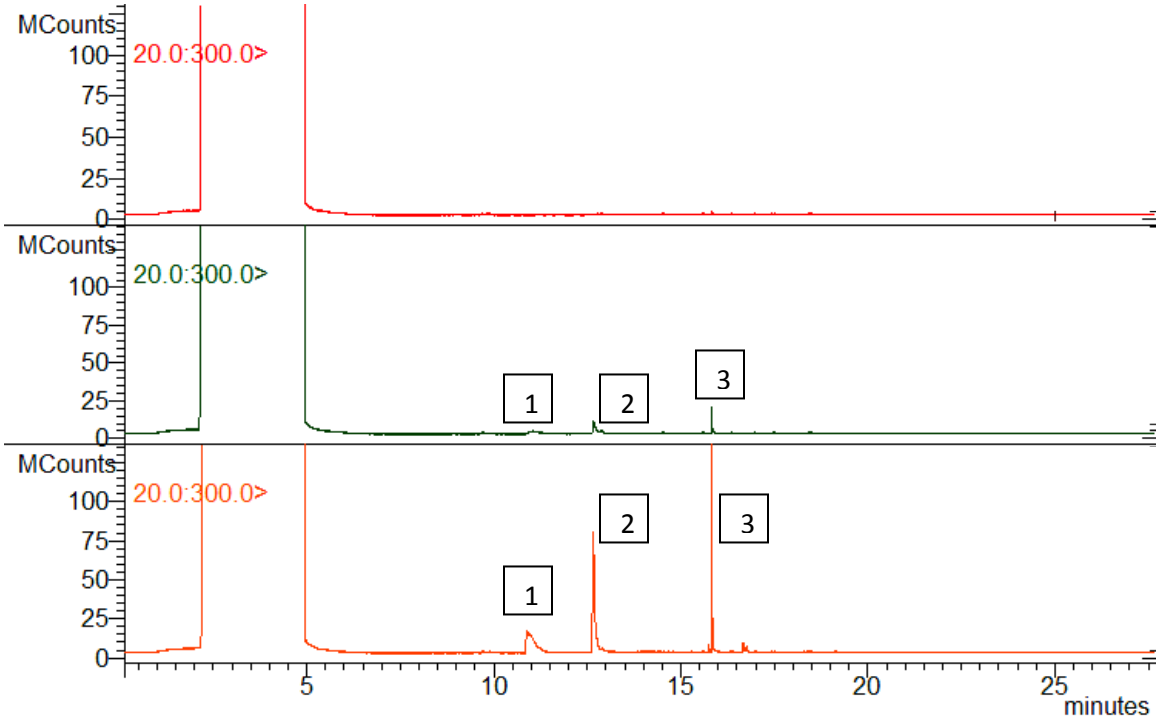


Figure 4.1 Chromatographic plots of volatiles produced by 10 adult *T. castaneum* after (top) 24 h, (middle) 48 h and (bottom) 72 h kept at 35°C

1 - Methyl 1, 4-benzoquinone 2 - Ethyl 1, 4-benzoquinone 3 - 1-Tridecene

The volatiles Methyl-1, 4-benzoquinone and Ethyl-1, 4-benzoquinone were reported as defensive secretions by Villaverde et al. (2007), because the compounds collected by them were obtained by agitating the insects and the volatiles were collected using SPME fiber. In another study conducted on dehusked rice involving *T. castaneum,* the same two benzoquinone derivatives were reported and considered to be secretions of *T. castaneum* and the study did not have any agitation or any other disturbance to the insects.

From the previous results we can confirm that the volatile organic compounds Methyl-1, 4-benzoquinone, and Ethyl-1, 4-benzoquinone were coming from *T. castaneum*. The 1-Tridecene addition to these two benzoquinone derivatives can be considered as a sex pheromone as reported by Geiselhardt et al. (2008).

4.3 C. *ferrugineus* in Wheat Flour and in Wheat

The *C. ferrugineus* insects placed in the vial with wheat and wheat flour did not produce any volatiles even at high insect density of 100 adult insects per vial. The previous studies for collecting the volatiles produced by *C. ferrugineus* have observed 4,8-dimethyl-(E,E)-4, 8 decadienolide (I) and (32,l lS) dodecen-I I-olide in picogram levels and the extraction were done over a period of 24 h with high insect density (Wong et al., 1983). The amounts observed were less than a few nanograms. The results obtained in our study using an auto sampler to extract the volatiles for just 20 minutes, did not show any volatile organic compounds even in trace levels.

4.4 Combination of *T. castaneum* and *C. ferrugineus*

T. castaneum adults are ten times larger in mass compared to *C. ferrugineus*, so we transferred fifty *C. ferrugineus* and five *T. castaneum* insects inside the vials. The volatile profile obtained were the compounds of *T. castaneum*. We did not get any peculiar compounds when both the insects were placed in the empty vial, wheat as well as in wheat flour. The chromatographic results of the *C. ferrugineus* and *T. castaneum* are shown in Figure 4.2.

4.5 Insects at Cold Temperature

The *T. castaneum and C. ferrugineus* adults were placed separately inside the empty vials and with wheat flour present, the results observed in the samples of *T.castaneum* were similar to the compounds reported in the ambient temperature studies, but the amount of volatiles produced was much higher. The results are shown in Figure 4.3 and in Table 4.2. The concentration of three compounds, Methyl 1-4,benzoquinone; Ethyl-1,4-benzoquinone; and 1-Tridecene produced by ten *T. castaneum* insects at minus 10°C were 30, 50, and 250 µg/100 µL whereas the concentration of the same three compounds produced by 10 insects at 35°C were 8.5, 9.1, and 10.6 µg/100 µL.

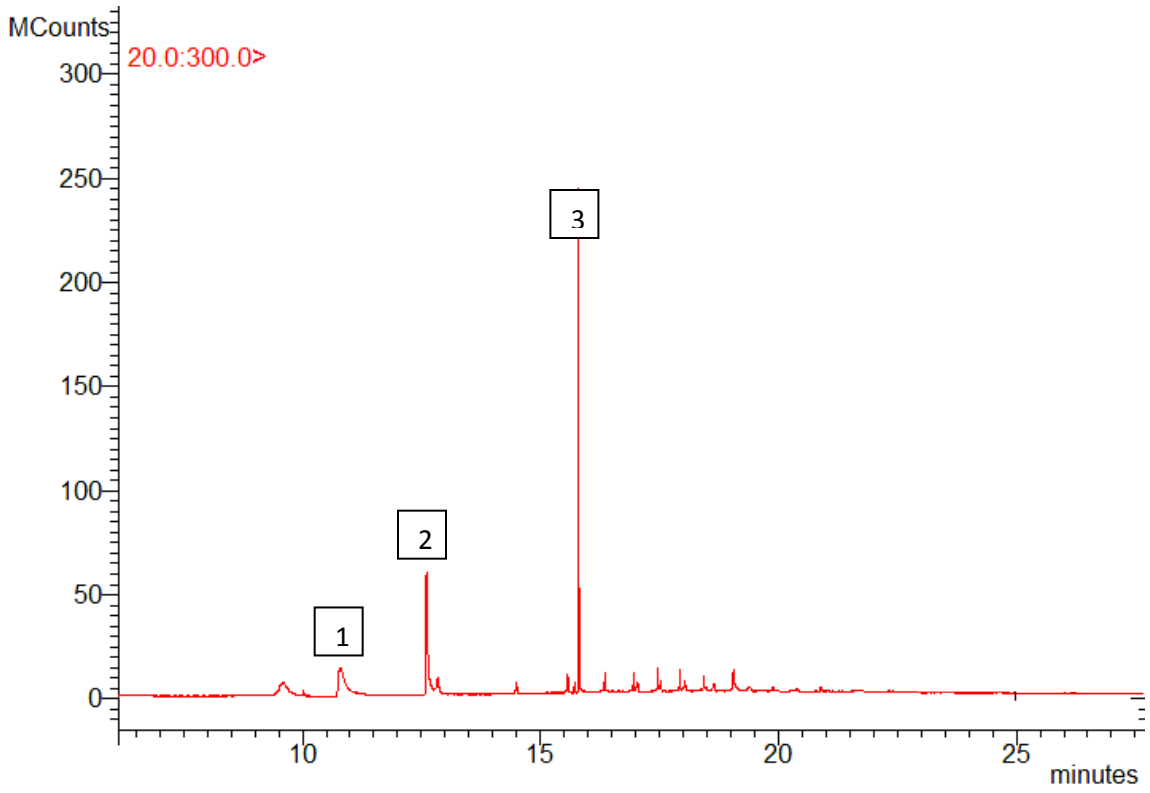


Figure 4.2 Chromatographic plot of volatiles produced by the combination of *C. ferrugineus* and *T. castaneum* adults at 35°C

1 - Methyl 1, 4-benzoquinone 2 - Ethyl 1, 4-benzoquinone 3 - 1-Tridecene

Table 4.2 Amount of volatiles produced by *T. castaneum* adults after being held at -10°C for 1 h in units of area under the peak or concentration (µg/100 µL)

No of Insects	MBQ		EBQ		1-Tridecene	
	Area under peak	Concentration (µg/100 µL)	Area under peak	Concentration (µg/100 µL)	Area under peak	Concentration (µg/100 µL)
10	3.976E+9	30	7.063E+9	50	5.995E+9	250

MBQ-Methyl 1-4, benzoquinone, EBQ-Ethyl 1, 4-benzoquinone

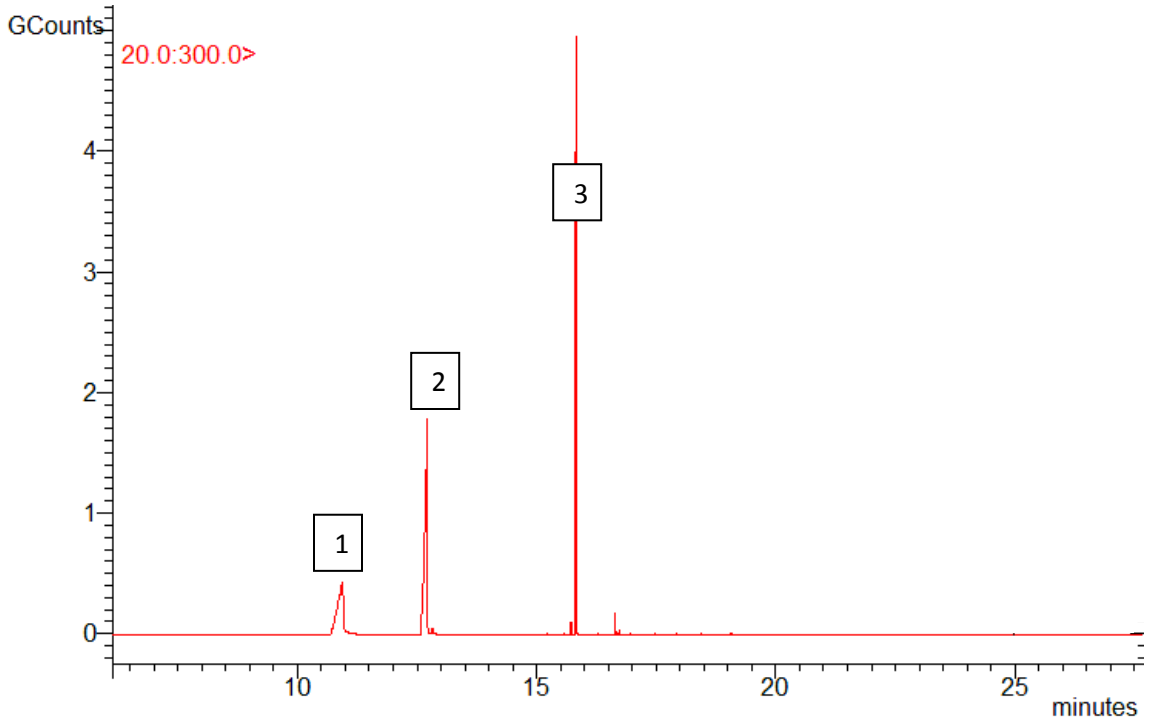


Figure 4.3 Chromatographic plot of volatiles produced by ten adult *T. castaneum* insects kept at minus 10°C

1 - Methyl 1, 4-benzoquinone 2 -Ethyl 1, 4-benzoquinone 3 - 1-Tridecene

The amount of volatiles produced at minus 10°C can be attributed to the fact that the cold condition was not favorable to the insects and they were stressed, so the insects produced enormous amount of volatiles.

4.6 T. castaneum adults with CWRS Wheat

The amount of volatiles released by red flour beetles placed in a vial filled with 15% moisture content wheat increased with an increase in the number of insects. The amount of volatiles released by three red flour beetles was less than the volatiles released by five red flour beetles (Figure 4.4).

The amount of volatiles generated by insects increased with the increase in time, three red flour beetles immediately after transferring them to the vials produced smaller amounts of volatiles than after 1 h (Figure 4.5). The amount of volatiles increase may be because of the new wheat substrate differed from the previous wheat flour used for rearing the insects for this study. The amount of volatiles increased up to 8 h and stabilized after that.

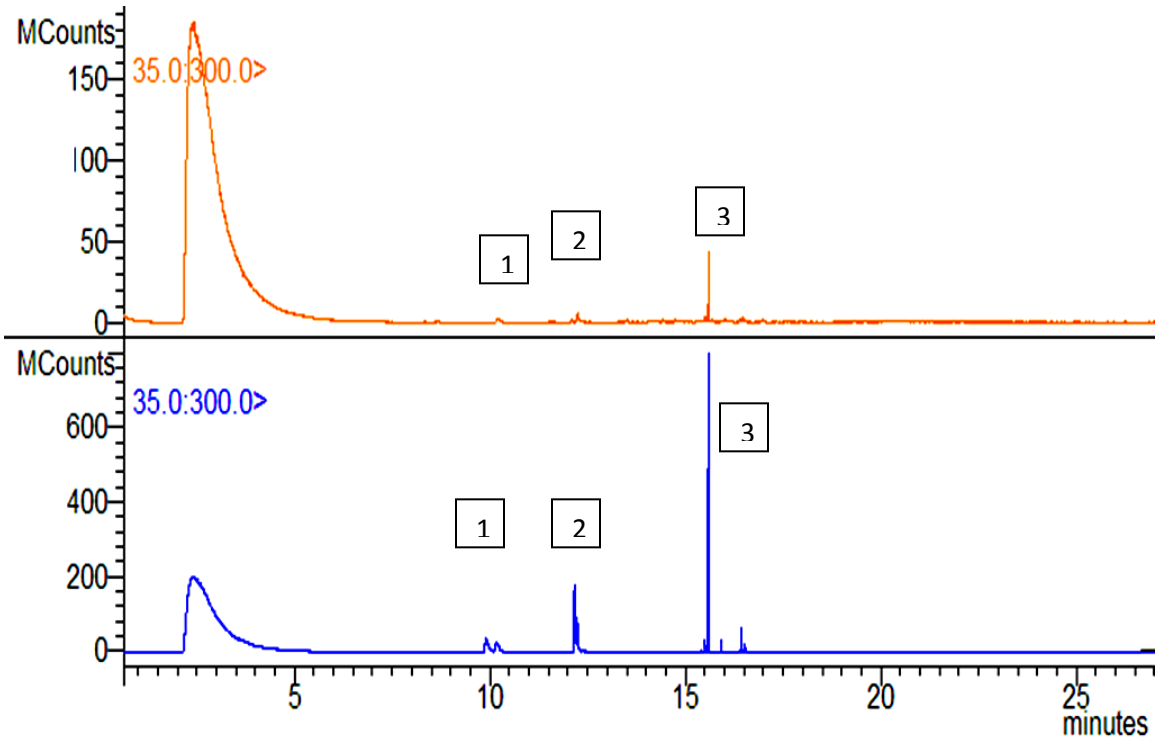


Figure 4.4 Chromatograms of the 15% moisture content wheat and three *T. castaneum* adults (top) and 15% moisture content wheat and five *T. castaneum* adult insects (bottom)

1 - Methyl 1, 4-benzoquinone 2 - Ethyl 1, 4-benzoquinone 3 - 1-Tridecene

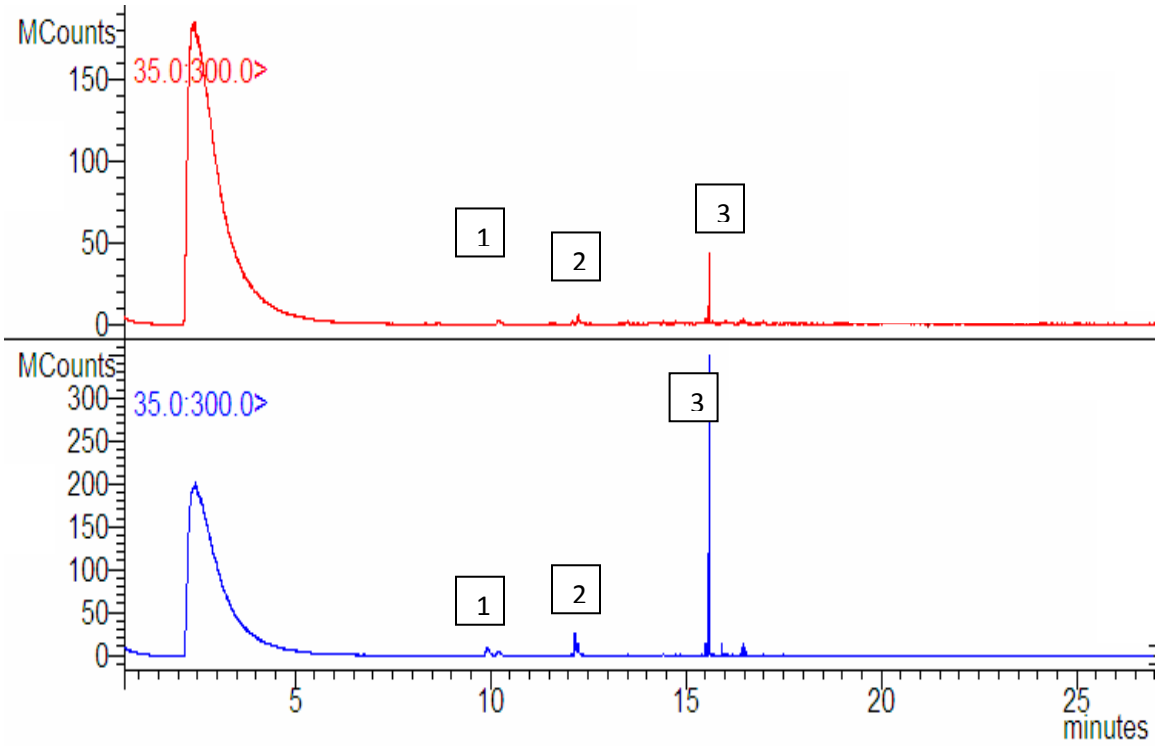


Figure 4.5 Chromatograms of the 15% moisture content wheat and three *T. castaneum* adults immediately after transferring them to vials (top) and after 1 h (bottom).

1 - Methyl 1, 4-benzoquinone 2 - Ethyl 1, 4-benzoquinone 3 - 1-Tridecene

4.7 *T. castaneum* larvae

The larvae of *T. castaneum* collected from the cultures did not produce any volatiles at 35°C. The number of larvae used was 15. Increasing the temperature to 45°C killed the larvae but did not produce any volatiles. This non production of volatiles can be attributed to lack of development of glands that produce volatiles.

4.8 Insects Heated to Higher Temperature

The *T. castaneum* adults kept in empty vials and preheated to 60°C produced similar large amounts of volatiles as were produced by the same species when kept at minus 10°C presumably from the stress of dying. *C. ferrugineus* did not produce any volatiles even after heating the vials to 60°C.

CHAPTER 5

5. CONCLUSION

The results show that the auto sampler, when optimized to the required extraction strokes of 20 at 1000 µL per stroke at the speed of 100 µL/s is a promising way of collecting volatiles from a headspace above wheat with insects. The feasibility of an automatic headspace sampler for the headspace analysis of stored grain insects is positive.

The volatile organic compounds produced by adult *T. castaneum* were Methyl-1, 4-benzoquinone; Ethyl-1, 4-benzoquinone; and 1-Tridecene. Among the three compounds, the amount of Ethyl-1, 4-benzoquinone was higher in amount compared to Methyl-1, 4-benzoquinone and increased with increase in number of insects, whereas the1-Tridecene was less than Methyl-1, 4-benzoquinone; Ethyl-1, 4-benzoquinone for five adult insects and was higher in amount than the other two compounds for 10 adult insects, and this inconsistency can be attributed to the increase in number of male insects inside the 10 insects sample.

The amount of volatile organic compounds produced by *T. castaneum* adults vary with insect densities. The concentration Methyl-1, 4-benzoquinone;

Ethyl-1, 4-benzoquinone; and 1-Tridecene produced by ten insects were 8.5, 9.1, and 10.6 µg/100 µL compared to 7, 8, and 4.2 µg/100 µL produced by five adult insects when placed in the wheat flour at ambient conditions. Insects exposed to high and low temperatures(60 and -10°C) produced very high amounts of volatiles compared to insects kept at 35°C possibly because of the stress of dying.

The *C. ferrugineus* adults did not produce volatiles in detectable levels even after 3 days in vials.

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APPENDIX

Chromatographic Plots of Volatiles Produced by Insects.

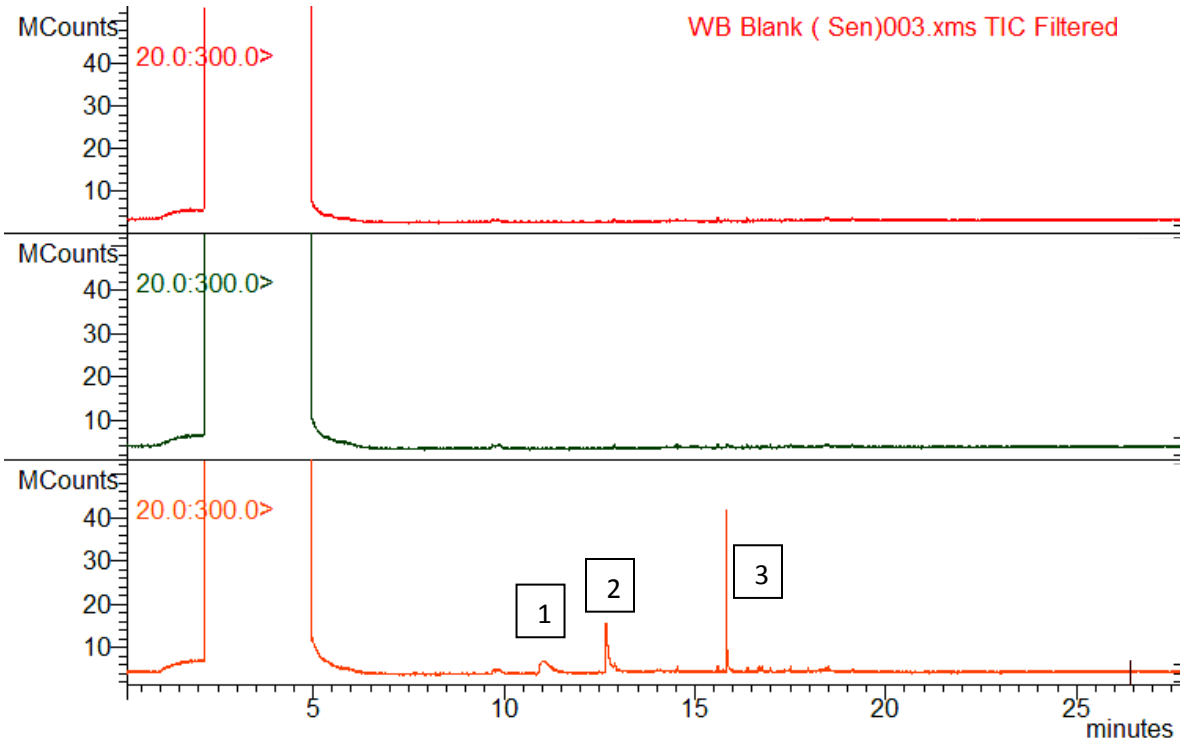

Figure A 1. Chromatographic plot of volatiles produced by 5 adult *T. castaneum* after (top) 24 h, (middle) 48 h, and (bottom) 72 h kept at 35°C

1 - Methyl 1, 4-benzoquinone 2 - Ethyl 1, 4-benzoquinone 3 -1-Tridecene

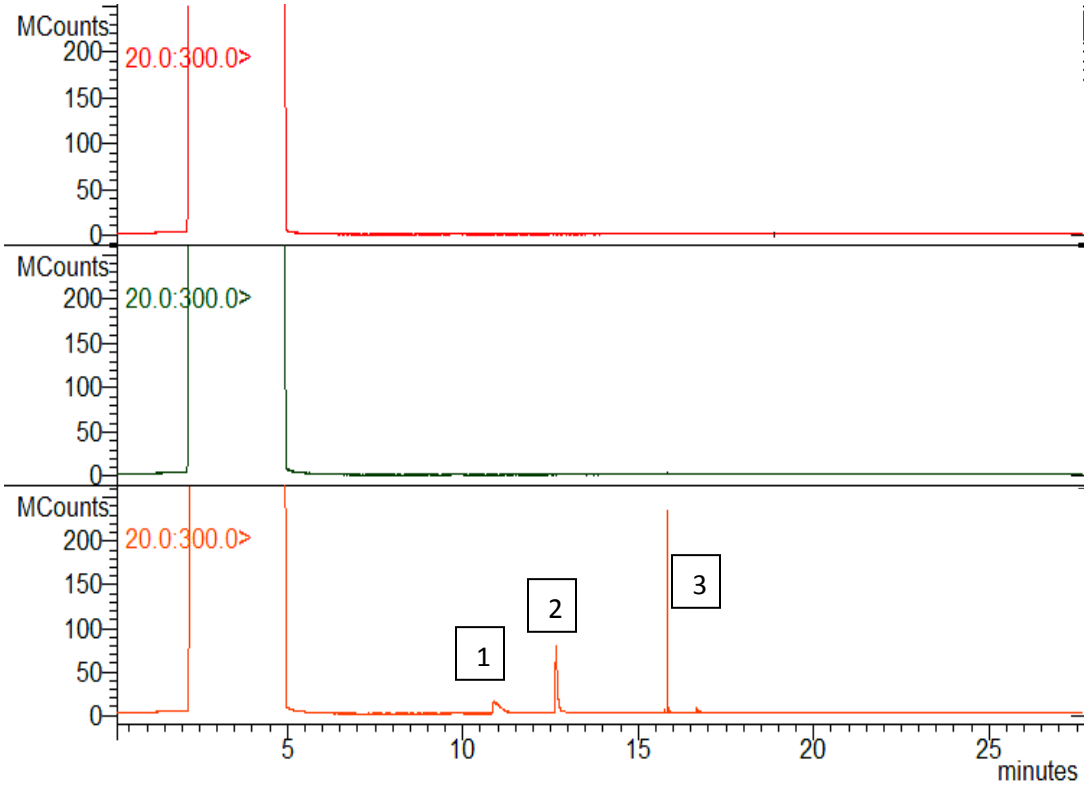

Figure A 2. Chromatographic plot of volatiles produced by 10 adult *T. castaneum* after (top) 24 h, (middle) 48 h, and (bottom) 72 h kept at 35°C

1 - Methyl 1, 4-benzoquinone 2 - Ethyl 1, 4-benzoquinone 3 - 1-Tridecene

Table A 1. Methyl-1, 4-benzoquinone

Common Name	Structure	Molecular Weight
Methyl-1, 4-benzoquinone	$C_7H_6O_2$	122

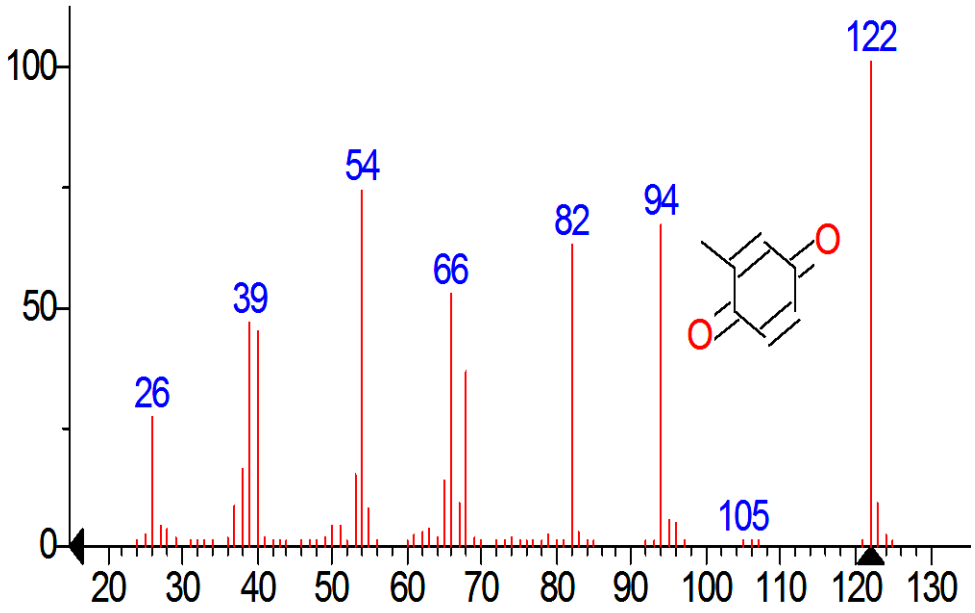

Figure A 3. Mass spectrum of methyl-1, 4-benzoquinone

Table A 2. Ethyl-1, 4-benzoquinone

Common Name	Structure	Molecular Weight
Ethyl-1, 4-benzoquinone	$C_8H_8O_2$	136

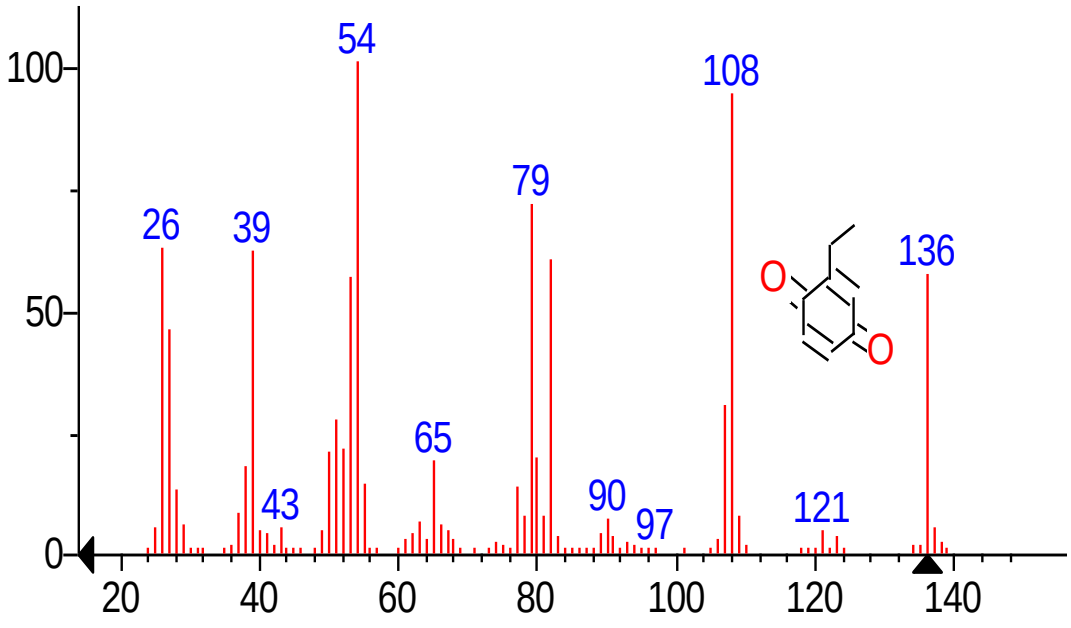

Figure A 4. Mass spectrum of ethyl-1, 4-benzoquinone

Table A 3. 1-Tridecene

Common Name	Structure	Molecular Weight
1-Tridecene	$C_{13}H_{26}$	182

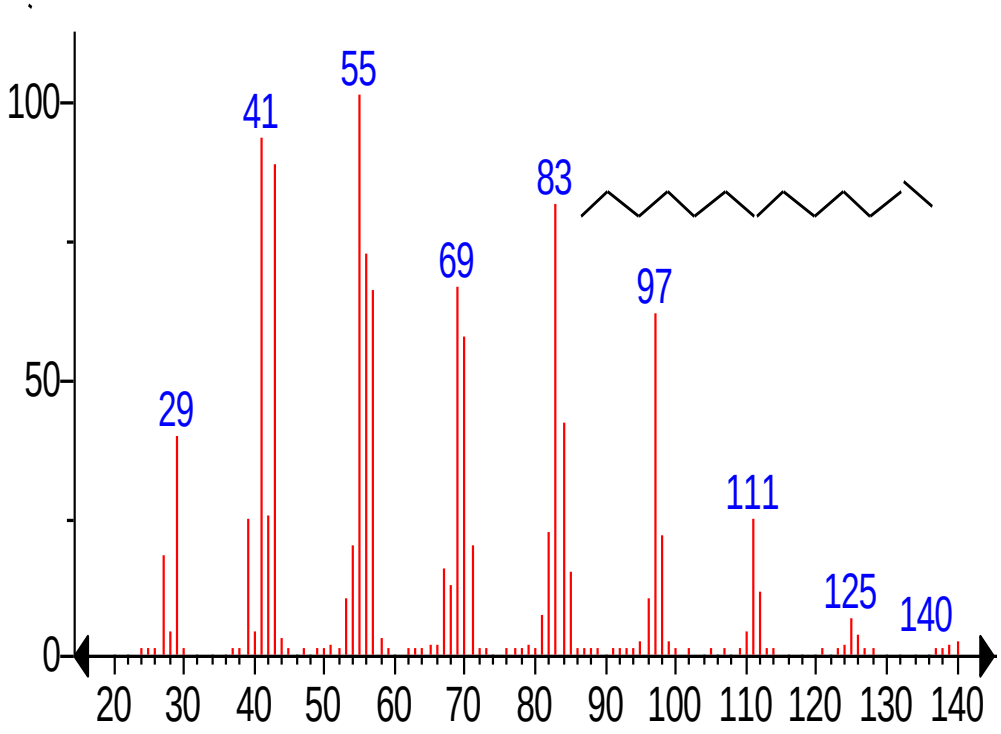

Figure A 5. Mass spectrum of 1-Tridecene

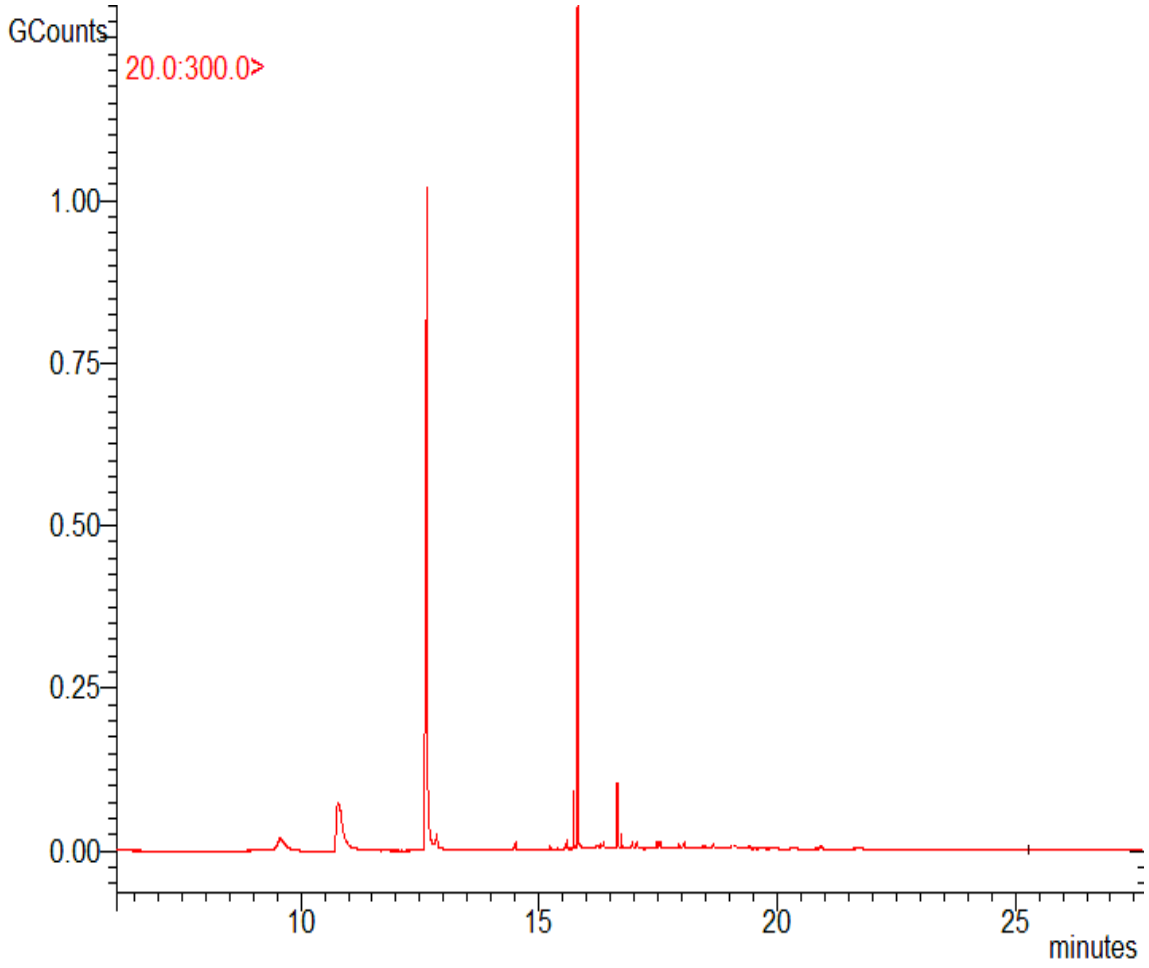

Figure A 6. Chromatogram of volatiles produced by 10 *T. castaneum* adults at 60°C

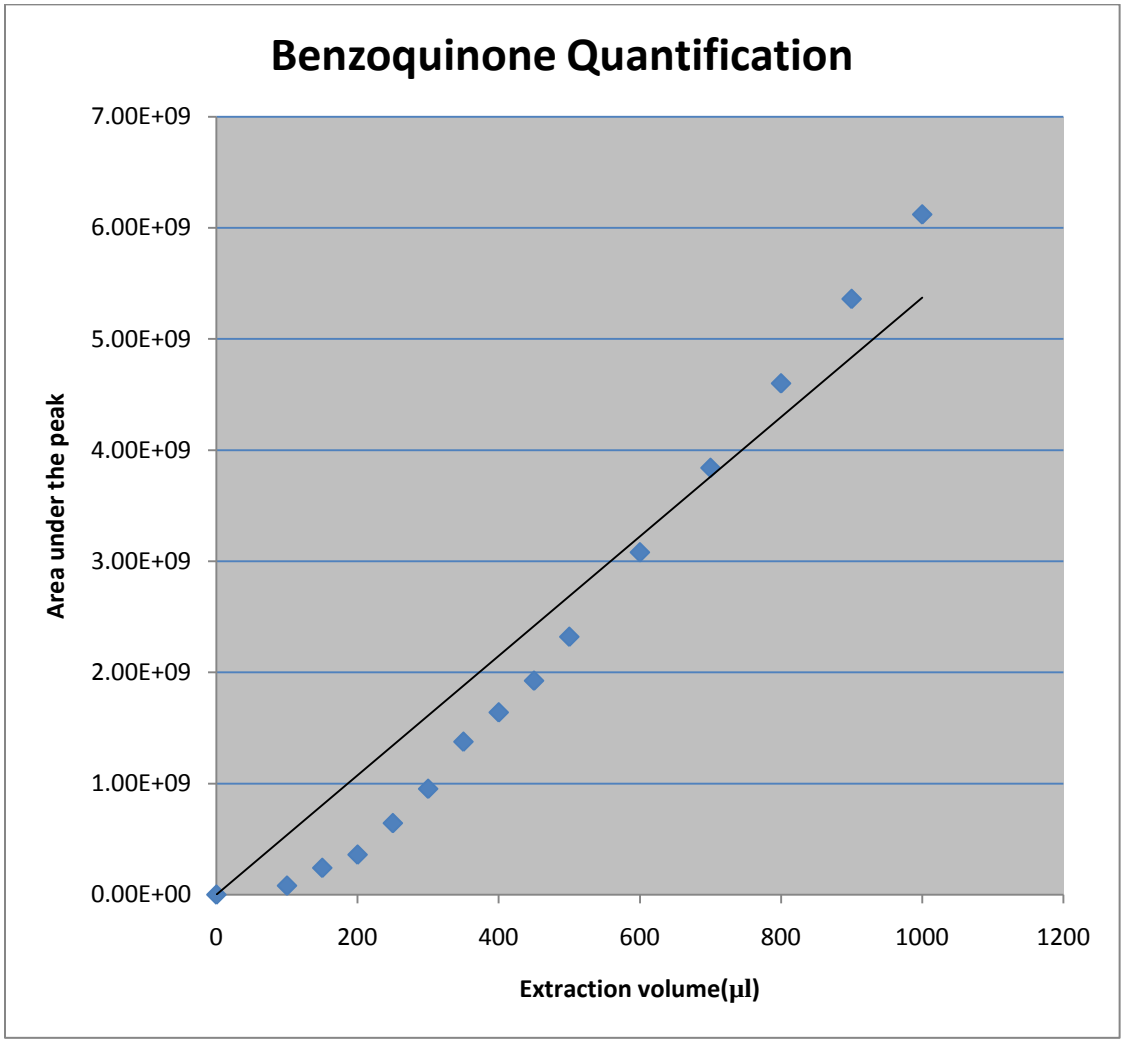

Figure A 7. Quantification of benzoquinone (extraction volume vs. area under the peak)

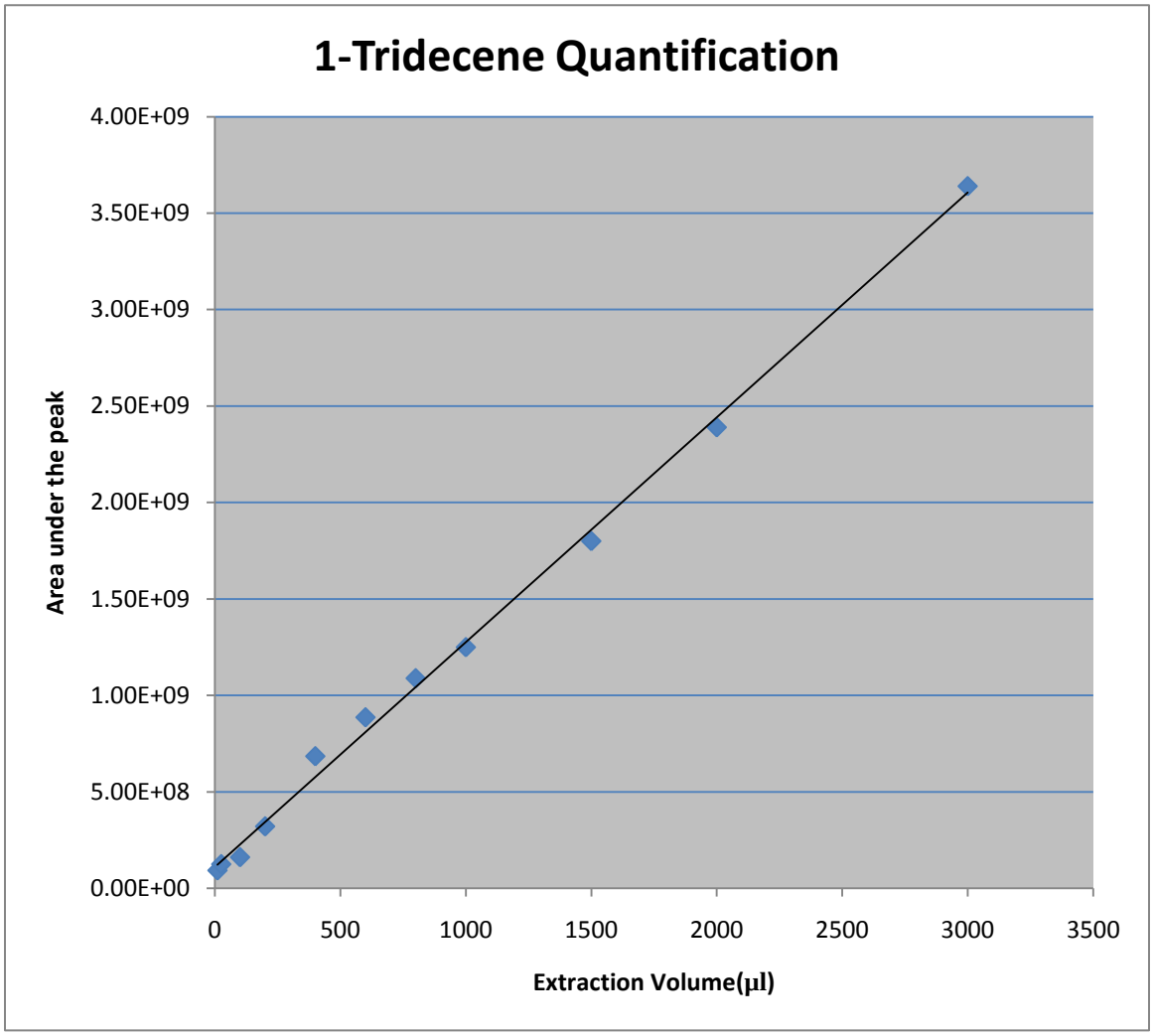

Figure A 8. Quantification of 1-Tridecene (extraction volume vs area under the peak)